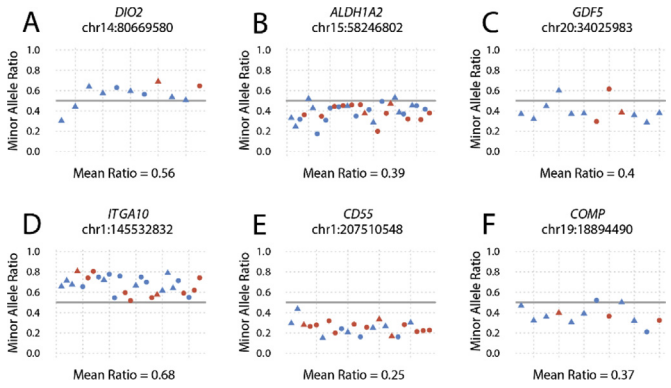
Among the 493 genes, we observed the reported AI events in OA susceptibility genes (e.g. ALDH1A2, DIO2, GDF5, figure 1), as well as a substantial number of novel AI events in cartilage expressed genes (e.g. PTGES3P1, CHST6, ITGA10, CD55, figure 1). Next, to gain disease relevant insight in the direction of the observed AI, we addressed differential gene expression between preserved and paired lesioned cartilage. Among the 11955 aligned Ensembl identifiers that passed quality control, 549 and 158 were respectively significantly (FDR<0.05) down (mean FC<0.667) and up (mean FC>1.5) regulated in lesioned compared to paired preserved cartilage. Among the total 707 genes that were differentially expressed, 31 were subject to AI marked by 71 SNPs. As proof of principle, we here present AI of the COMP gene, detected, among others, in heterozygous carriers of rs4808155 (figure 1) which is in full linkage disequilibrium (D'=1) with rs8104411, a previously reported OA susceptibility SNP (Ramos et al. 2014). Furthermore, COMP was significantly up regulated in lesioned cartilage (FDR<0.005, FC>1.5).

Conclusions: By confirming earlier reports and identifying new genes likely involved in OA disease progression, we here demonstrate the power of next generation RNA sequencing. In this respect, the 71 observed genetic variants that confer AI in the 31 genes that additionally mark the OA disease process by differential gene expression, are likely to be causally or protectively involved in the complex OA pathophysiology of articular cartilage, as they stress or attenuate the observed differential expression of the respective genes. Although these genetic variants do bear transcriptomic functionality in disease relevant tissue, additional functional approaches in cell or animal models are necessary to further characterize such causal relationships.



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DEMETHYLATION OF AN NUCLEAR FACTOR-KB (NF-KB) ENHANCER ELEMENT ORCHESTRATES INOS INDUCTION IN OSTEOARTHRITIS VIA CELL CYCLE REGULATION

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Purpose: Osteoarthritis (OA) is a complex disease of the joint, characterized by progressive degradation of the cartilage matrix by aggrecanases and collagenases. In normal adult cartilage, chondrocytes present, in the non-stressed steady state, as quiescent cells demonstrating negligible turnover of the cartilage matrix. In contrast, in OA, the chondrocytes become "activated", characterized by cell proliferation, cluster formation, and the increased production of matrix proteins and matrix-degrading enzymes. Nitric oxide, the product of inducible nitric oxide synthase (iNOS), not only suppresses the synthesis of cartilage matrix, but also increases expression of proteases in OA and we have recently described that demethylation of the NF-κB enhancer region at −5.8 kb of the iNOS gene leads to increased transcription through elevated binding of this transcription factor. The current study has examined the methylation profile of the NF-κB enhancer region at −5.8 kb of iNOS and the subsequent role in the induction of OA via cell cycle regulation.

Methods: Human articular cartilage samples were obtained from 12 patients following hemiarthroplasty as a consequence of femoral neck fracture (NOF) and from 16 OA patients who underwent total hip arthroplasty. Isolated primary human chondrocyte samples (n = 7) were cultured for 5 weeks in 2 groups: (i) cultured without treatment (control culture) and (ii) cultured using 2 μM 5-azadeoxycytidine (5-aza-dC). Percentage methylation was determined by pyrosequencing, gene expression by qPCR and cell proliferation was determined using the MTT assay. Transient transfections using the chondrocytic cell line C28/I2 were induced to determine the effect of the NF-κB enhancer region on cell proliferation and the influence of DNA methylation. Cell cycle distribution was analysed by fluorescence-activated sorting (FACS).

Results: Analysis of several NF-κB enhancer elements localised upstream of the −5.8 kb of the iNOS gene showed that this element is crucial for induction of the human iNOS promoter in articular chondrocytes. In vitro de-methylation with 5-aza-dC showed decreased levels of DNA methylation at CpG sites localised at −5.8 kb in articular chondrocytes, which correlated with higher levels of iNOS expression. In vitro methylation of the NF-κB enhancer region at −5.8 kb increased the percentage of cells at G0/G1 cell cycle phase. Loss of methylation within this region correlated with, enhanced proliferation and increased number of cells at G2/M phase. OA chondrocytes demonstrated upregulation of the G0/G1 cell cycle progression markers Cyclin D1 and CDK6 in contrast to control cells.

Conclusions: We demonstrate the loss of methylation that occurs at specific CpG sites localised at the −5.8 kb NF-κB enhancer region of the iNOS gene in OA chondrocytes permits the binding of this transcription factor activating the expression iNOS. This results in subsequent altered cell cycle regulation of OA chondrocytes and altered proliferative phenotype and transmission of the pathogenic phenotype to daughter cells. This study indicates that inhibition of cell cycle progression by iNOS enhancer hypermethylation is capable of reducing pro-inflammatory responses via down-regulation of NF-κB with important therapeutic implications in OA.

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EPIGENETIC REGULATION OF INTERLEUKIN-8, AN INFLAMMATORY CHEMOKINE, IN OSTEOARTHRITIS

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Purpose: Involvement of inflammation in the development and progression of OA has been implicated. Interleukin 8 (IL-8) is an inflammatory chemokine, involved in the inflammatory process and the pathophysiology of OA. Importantly, within IL8, the 1000-bp of the proximal promoter region contains only three CpG sites all located close to transcriptional binding sites for NF-κB, AP-1 and C/EBP. However, the methylation status of these CpG sites and subsequent involvement in the regulation of IL8 regulation remains, to date, unknown. The current study has examined whether the increased expression of IL8 in human OA is a consequence of epigenetic regulation, specifically DNA hypomethylation.

Methods: Human articular cartilage samples were obtained from 15 patients following hemiarthroplasty as a consequence of femoral neck fracture (NOF) and from 15 OA patients who underwent total hip arthroplasty. IL8 expression levels and the percentage CpG methylation were quantified by qRT-PCR and pyrosequencing to compare OA patients with non-OA controls. Isolated primary human chondrocyte samples (n = 7) were cultured for 5 weeks in 2 groups: (i) cultured without treatment (control culture) and (ii) cultured using 2 μM 5-azadeoxycytidine (5-aza-dC). The effect of CpG methylation on IL8 promoter activity was determined using a CpG-free vector; co-transfections with expression vectors encoding NF-κB, AP-1 and C/EBP were subsequently undertaken to analyse for IL8 promoter activity in response to changes in methylation status. Furthermore, to determine the CpG sites critical for IL8 promoter activity, we compared IL8 wild type promoter construct activity against 6 vectors containing mutations at different CpG sites.

Results: IL8 expression in OA patients was 37-fold higher than in NOF patients. OA chondrocytes displayed a 22%, 26% and 15% statistically significant (P<0.01) reduction in methylation status at the -116, -106 and -31 CpG sites respectively. Multiple regression analysis revealed that the degree of methylation of the CpG site located at −116-bp was the strongest predictor of IL8 expression. In addition, advanced age and OA were associated with higher IL8 expression. IL8 expression by chondrocytes cultured using 5-aza-dC was observed to be 4.0-fold higher than in control culture chondrocytes. Significantly, the

percentage methylation of the CpG site located at −116-bp in 5-aza-dC culture was lower than that in control culture. In vitro DNA methylation was noted to decrease IL8 promoter basal activity. Furthermore, NF-κB, AP-1 and C/EBP strongly enhanced IL8 promoter activity whilst DNA methylation inhibited the effects of these three transcription factors. Finally, point mutations created on any single CpG site or two CpG sites resulted in a significant increase in IL8 promoter activity by 3.4 to 5.4 fold.

Conclusions: The current study demonstrates that the increased expression of IL8 in human osteoarthritic chondrocytes is regulated by DNA demethylation in cooperation with transcription factors. We show for the first time that the percentage methylation of specific CpG sites correlates with IL8 gene expression level in clinical OA samples. Furthermore, DNA methylation of specific CpG sites appears to be a basal repression mechanism of IL8 expression. These findings offer a potential predictive marker and a putative target, in this inflammatory chemokine, for pharmacological intervention in the treatment of OA and, potentially, other arthritic diseases.

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RUNX2 EXPRESSION IS REGULATED BY METHYLATION STATUS OF SPECIFIC CPG SITES IN THE P1 PROMOTER AND CORRELATES WITH MMP13 EXPRESSION IN HUMAN OSTEOARTHRITIC CHONDROCYTES

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Purpose: Runt-related transcription factor 2 (RUNX2) also known as core-binding factor subunit alpha-1 belongs to the family of runt domain transcription factors. RUNX2 is essential for the maturation of osteoblasts and both intramembranous and endochondral ossification. In the articular cartilage, RUNX2 regulates MMP13 expression and is involved in the pathophysiology of osteoarthritis (OA). However, transcriptional regulation mechanism of RUNX2 in articular chondrocytes is far from clear. RUNX2 has two major isoforms. Type II isoform is restricted to osseous cells and mature chondrocytes, and the transcription is initiated at the distal promoter P1. Importantly the RUNX2 P1 promoter has a fragment containing a 600bp of sequence upstream of the transcription start sufficient for promoter activity, with only a few CpG sites contained in this region. We hypothesized that methylation status at specific CpG sites in the RUNX2 P1 promoter predominantly regulate promoter activity and correlate with messenger RNA levels in human chondrocytes.

Methods: The superficial and deep zone of cartilage from patients with a fracture of the neck of femur (NOF; n = 11) were used for isolation of non-OA/healthy chondrocytes, whereas cartilage pieces adjacent to weight-bearing areas of OA femoral heads (lacking surface zones) were harvested to obtain OA chondrocytes (n = 15). Gene expression levels and percentage methylation of CpG sites within the RUNX2 P1 promoter were quantified by qRT-PCR and bisulfite pyrosequencing. Isolated primary human chondrocyte samples (n = 6) were cultured for 5 weeks in 3 groups: (i) cultured without treatment (control culture) and (ii) cultured using 2 μM 5-azadeoxycytidine (5-aza-dC). The effect of CpG methylation on RUNX2 promoter activity was determined using a CpG-free vector. Furthermore, to determine the CpG sites critical for RUNX2 promoter activity, RUNX2 wild type promoter construct activity was compared against 4 vectors containing mutations at different CpG sites.

Results: RUNX2 expression in OA and NOF deep chondrocytes were 34-fold ($p < 0.01$) and 79-fold ($p < 0.01$) higher compared to NOF superficial chondrocytes. OA chondrocytes displayed a 10%, 14%, 32% and 41% statistically significant ($P<0.01$) reduction in methylation status at the −720, −686, −336 and +17 CpG sites respectively, whereas NOF deep chondrocytes a 13% and 19% reduction at the -336 and +17 CpG sites. A significant negative correlation was observed between RUNX2 gene expression and the percentage methylation of the CpG sites in OA chondrocytes. MMP13 expression in OA chondrocytes was 27-fold and 89-fold higher than observed n NOF superficial and deep chondrocytes, respectively. A significant positive correlation was observed between RUNX2 and MMP13 gene expressions in OA chondrocytes. RUNX2 expression by chondrocytes cultured using 5-aza-dC was observed to be 3.3-fold higher than in control culture chondrocytes. Significantly, the percentage methylation of the CpG site located at −336-bp in 5-aza-dC culture was lower than observed in control culture (55% vs. 87%). In vitro DNA methylation was noted to decrease RUNX2 promoter activity. Point mutations (CG to TG) created at the −336 and +17 CpG sites resulted in a significant reduction in RUNX2 P1 promoter activity.

Conclusions: The current studies show that the increased expression of MMP13 in human osteoarthritic chondrocytes is correlated with RUNX2 expression. Critically, we show, methylation status of specific CpG sites in the P1 promoter is a key regulator of RUNX2 expression in human chondrocytes. These studies show for the first time that the percentage methylation of specific CpG sites correlates with RUNX2 gene expression level in clinical OA samples. Furthermore, point mutations created at the −336 and +17 CpG sites resulted in a significant reduction in RUNX2 P1 promoter activity. These findings indicate that CpG sites located at −336 and +17 are crucial regulators of RUNX2 transcription and offer a putative target for pharmacological intervention in the treatment of OA.

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THE MTDNA HAPLOGROUPS INFLUENCE THE RADIOGRAPHIC PROGRESSION OF KNEE OSTEOARTHRITIS. DATA FROM CHECK REPLICATE OAI RESULTS

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Purpose: Significant influence of the mtDNA haplogroups on radiographic progression of knee OA (KOA) has been reported. For the present study, we investigated the influence of the mtDNA haplogroups on radiographic KOA progression in subjects of the CHECK (Cohort Hip and Cohort Knee) cohort, a running 10-year prospective multi-center cohort of subjects, with pain and/or stiffness of one and/or both knees

Methods: We assigned the mtDNA haplogroups to the entire CHECK cohort (n=1002 subjects). For this work, according to the criteria reported elsewhere (van Spil et al. Osteoarthritis Cartilage 2012;20:1278-85), we have selected subjects that did show radiographic KOA at baseline (K&L grade=1 for the left and/or right knee since maximum baseline K&L grade=1, in accordance with the early-stage OA in CHECK subjects), reaching a total of n=417 subjects. Progression in these subjects was defined as an increase of ≥1 K&L grade for the left and/or right knee during 5 years follow-up period.

Appropriate statistical analyses, including chi-square contingency tables, logistic regression models adjusting by gender, age, body mass index (BMI), bilateral KOA and total WOMAC at baseline, as well as Receiver Operating Curve (ROC) with their respective Area Under the Curve (AUC), were carried out using SPSS software (v.19)

Results: The 417 subjects that met the eligibility criteria to study radiographic progression included 81.1% females and 18.9% males, they were older than 45 years (mean age: 56.24±5.07 years; range: 45-66) and had a mean BMI of 26.80 Kg/m2 (range: 15.43-48.85). Chi-square analysis revealed that the mtDNA haplogroup T significantly associated with lower risk of radiographic progression (OR=0.467; CI=0.250-0.874; p=0.015); analysing by mtDNA clusters, the cluster TJ significantly associated with lower risk of progression too (OR=0.586; CI=0.366-0.939; p=0.025). By comparing each of the mtDNA haplogroups with the most common haplogroup H in the regression model, we detected that carriers of the mtDNA haplogroup T were at lower risk for K&L grade progression (31.4% progressors) than carriers of the most common mtDNA haplogroup H (50.6% progressors) (OR=0.414; CI=0.204-0.844; p=0.015); in terms of mtDNA clusters, subjects in cluster TJ were at lower risk of progression than subjects in the cluster HV (OR=0.551; CI=0.317-0.955; p=0.034). Additionally, either including mtDNA haplogroups or mtDNA clusters in the regression model, both BMI (OR=1.064; CI=1.010-1.120; p=0.020) and bilateral KL=1 at baseline (OR=3.595; CI=2.345-5.510; p<0.001) were also significantly associated with an increased risk of radiographic progression of KOA attending to K&L grade. The AUC of this regression model was 0.723 and 0.722 after including either haplogroups or clusters respectively.

Conclusions: This work strengthens the hypothesis that mitochondrial genome is a key factor in the progression of the KOA disease. As noted in KOA patients of the OAI cohort, the early identification and classification of patients with haplogroup T and the most common haplogroup H would permit to identify those patients more prone to a rapid radiographic progression of the disease.